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In vitro short-term effects of SMS 201-995, bromocriptine and TRH on growth hormone cell morphology from human pituitary adenomas

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Summary. This study reports, by immunocytochemistry, ultrastructure and morphometry, the *in vitro* effects of SMS 201-995 (10 nM), bromocriptine (1 μM) and TRH (10 μM) on the morphology of cells from two acromegalic patient adenomas containing immunoreactive growth hormone (GH). By electron microscopy, one tumor presented numerous large secretory granules (densely granulated growth hormone cell adenoma) while they were scarce and small in the other (sparsely granulated growth hormone cell adenoma); fibrous bodies could be seen in the specimen and *in vitro.* In the sparsely granulated growth hormone cell adenoma, TRH produced an increase in endoplasmic reticulum surface density compared to the other cultures. Bromocriptine increased the number and decreased the secretory granule diameters, while SMS 201-995 produced no significant changes in the same time.

In the densely granulated growth hormone cell adenoma, the three substances increased the number of granules. TRH increased the mitochondrial volume density and endoplasmic reticulum surface density (with respect to the other cultures). SMS 201-995 decreased the mitochondrial and lysosome volume densities and endoplasmic reticulum surface density. We conclude that 1) TRH produces in cultured cells of both adenoma types an increase in cellular activity. 2) In cultured sparsely granulated growth hormone adenoma cells, bromocriptine has a stronger inhibitory effect than SMS 201-995. In cultured densely granulated growth hormone cells adenoma, bromocriptine has smaller inhibitory effect than SMS 201-995

Key words: Acromegaly, Morphometric analysis, TRH, Bromocriptine, SMS 201-995

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Introduction

The physiological inhibitor of pituitary secretion of growth hormone (GH) in humans is somatostatin (Brazeau et al., 1973, 1974). This hypothalamic hormone also inhibits GH release in GH-producing pituitary adenomas both in vivo (Hall et al., 1973) and *in vitro* (Lamberts et al., 1985a,b).

Somatostatin *in vitro* increases the size and number of secretory granules inducing no ultrastructural changes in the nuclei, rough surfaced endoplasmic reticulum (RER), Golgi apparatus or mitochondria (Peillon et al., 1976). The octopeptide SMS 201-995 (analogous to somatostatin) has been proved to be a powerful inhibitor of GH secretion in acromegalic patients, producing a decrease in tumor size at the same time (Plewe et al., 1984; Lamberts et al., 1985a,b; George et al., 1987). Morphology of cells treated with SMS 201-995 confirms the inhibition of GH release (George et al., 1987).

Bromocriptine is a dopaminergic agonist that produces a strong decrease in GH secretion in acromegaly adenomas and also tumor size (Peillon et al., 1979; Spada et al., 1982; Oppizi et al., 1984). Presence of dopaminergic receptors in GH adenomatous cells was proved by Breisson et al. (1982) and subsequently characterized by Koga et al. (1987).

Tirotropin releasing-hormone (TRH) directly inhibits GH secretion in normal, foetal and adult pituitary tissue; in acromegaly, TRH paradoxically produces stimulation of GH *in vivo* and *in vitro*, although when there was no response *in vivo*, there was none *in vitro* either (Marcovitz et al., 1982). THR can be a physiological GH secretion regulator, the paradoxical effect in acromegaly perhaps being due to an alteration at tumor cell level (Marcovitz et al., 1982). Ishibashi and Yamaji (1985) found that TRH stimulates release of prolactin (PRL) in all prolactin cell adenomas studied, but its effects on GH secretion in acromegaly are less frequent.

This study reports the acute *in vitro* effects (4 hours)

in two GH-producing adenomas, of SMS 201-995, bromocriptine and TRH. The purpose of this study is to: 1) Analyse by immuocytochemistry and ultrastructure the first cellular changes following addition of these substances to the culture medium, and 2) devise a morphometric model in order to evaluate cellular modifications objectively.

Materials and methods

1. Patients

Case number 1: Male aged 40 with clinical signs of acromegaly. Normal opthalmological examination. Computed Tomography (CT) revealed a 14 mm diameter intrasellar mass. GH and PRL basal serum levels were 11.8 and 22.5 ng/ml respectively. The pituitary adenoma was removed via transsphenoidal surgery. Immunocytochemical diagnosis was positive for GH and negative for PRL.

Case number 2: Female aged 57 with evident clinical signs of acromegaly and very evident macroglosia. With no visual alterations, CT showed an intrasellar mass with discrete suprasellar growth. Serum levels of pituitary hormones were normal except for GH with basal values of 44.9 ng/ml. Stimulation test with GRF 1-29 and inhibition test with SMS 210-995 and bromocriptine were all positive. TRH produced a decrease of GH serum levels. No preoperatory treatment was performed. Pituitary adenomectomy was revomed transsphenoidally. Immunohistochemical diagnosis was positive for GH and negative for PRL.

2. Culture method

Adenomas were washed and sectioned in a solution of ClNa (0.7 M), ClK (26 mM), PO_4Na_2H-$12H_2O$ (2.8 mM), glucose (27 mM), HEPES (48 mM) and BSA (2 mg/ml) with a pH of 7.3 at 37° C and added to 100 I.U. of penicillin and 100 µg/ml of streptomycin. Cellular dispersion was performed in this solution with 0.1% collagenase (Type V, Sigma Chemical Co. USA) for 30 minutes. Cells were then counted simultaneously with a trypan blue exclusion viability test.

Monolayer cultures were done in Falcon flasks at 37° C with a concentration of 10^6 cells/ml in Eagle's Minimal Essential Medium in Earle's solution pH 7.3, supplemented with glutamine and 10% fetal calf serum, 100 I.U./ml of penicillin and 100 µg/ml of streptomycin.

Following 48 hours of culture they were renewed with fresh serum-free medium adding 10 nM of SMS 201-995, 10 µM of TRH and 1 µM of bromocriptine respectively. Four hours later, cultures were washed with phosphate buffer solution (PBS) and cells were removed with 0.2%, trypsin in PBS. Cells were transferred to Beem capsules and centrifuged at 100 xg for ten minutes for pellet. The cellular pellets and a portion of the tumor mass were fixed in 2% glutaraldehyde in PBS and postfixed in 1% osmium tetroxide in PBS. Following dehydration with ethanol they were embedded in Epon 812.

3. Morphological methods

Semithin sections were then taken of the resulting block for immunocytochemistry and ultrathin sections for ultrastructural and stereological study.

Following deplastification of the semithin sections with sodium metoxide, in order to detect the presence of GH and PRL they were stained with anti-hGH at 1:800 and anti-hPRL 1:800, 1:100, 1:10 (kindly supply by NIADDK, NIH).

Ultrathin sections were stained with uranyl acetate and lead citrate for study using a Jeol CX-200 microscope.

Sampling for stereological analysis was performed randomly considering the sample satisfactory when the standard error was lower than 10% of the mean.

Protocol on table 1 was followed for this analysis. On levels I, II and III, micrographs were projected on different test systems (Weibel and Bolender, 1973). Volume and numerical densities of the cytoplasmic organelles were calculated in relation to the cytoplasmic volume density. Test W of Shapiro and Wilk was applied to the sample extracted from each culture. Each stereological parameter was submitted to a variance test with a 95% significance level.

Between 400 and 500 culture granules were measured with an automatic image analyzer (IBAS II Kontron Co., West Germany). Variance analyses between all the treated groups and also between each treated group and control culture were performed with the Stat 2 program.

Results

1. Immunocytochemistry

Both adenomas were exclusively positive for anti-hGH, both specimen and all cultures.

2. Ultrastructure

2.1 Specimen

In case 1, large irregularly shaped cells with clear cytoplasm were found (Fig. 1). Nuclei were large and although the majority were rounded some irregularly shaped. Nucleoli were generally very evident. Mitochondria were large, abundant and with inverted cristae. Their RER was formed by small and abundant flattened cisternae although in some areas it tended to be arranged laminarly. The Golgi apparatus was not very evident. Fibrous bodies were sometimes observed near the nuclei. Few lysosomes were found. Secretory granules were scarce with a mean diameter of 119 nm (Table 2) most of them scattered over the periphery of the cytoplasm. No granule extrusion was found. Considering its ultrastructural and immunocytochemistry characteristics we concluded it was a sparsely granulated GH cell adenoma.

In case 2, cells with a dense cytoplasm were observed, with a more or less rounded shape, eccentric nuclei and a lower number of mitochondria than in case 1. RER was

Table 1. Protocol of electron microscopic morphometric analysis of growth hormone cell adenomas treated for 4 h with TRH, SMS 201-995 and bromocriptine.

LEVEL	PRIMARY MAGNIFICATION	STEREOLOGICAL TEST SYSTEM	PARAMETERS	
I	4000	Square lattice $P_T = 100$	Volume density of nuclei	Vvnu
			Volume density of cytoplasm	Vvci
			Volume density of cell	Vvce
II	6000	Double lattice 9 : 1	Volume density of mitochondria	Vvmi
			Volume density of lysosome	Vvly
III	12000	Multipurpose $P_T = 100$	Surface density of endoplasmic reticulum	Sver
			Surface density of Golgi apparatus	Svgo
			Surface density of mitochondria	Svmi
Automatic Image Analysis			Numerical density of secretory granules	Nvgr
			Mean diameter of secretory granules	Dm

Table 2. Electron microscopic morphometric analysis of sparsely granulated growth hormone cell adenoma treated for 4 h with TRH, SMS 201-995 and bromocriptine *in vitro*.

	LEVEL I			LEVEL II		LEVEL III			AUTOMATIC IMAGE ANALYSIS	
	Vvnu	Vvci	Vvce	Vvmi	Vvly	Sver	Svgo	Svmi	Nvgr	Dm(nm)
Control	16.6 ± 1	51.4 ± 4.1	68 ± 5.7	35.8 ± 2	4.1 ± 0.3	37.3 ± 2.7	23.6 ± 1.2	10.6 ± 1	34.2 ± 2.5	107 ± 6
TRH	10.9 ± 1	47.2 ± 3	58.1 ± 4.6	25.5 ± 1.6	6.4 ± 0.5	57.3 ± 4.5*+	20.9 ± 1.8	11.6 ± 1.1	31.3 ± 2	84 ± 4
SMS 201-995	15.5 ± 1.1	48.5 ± 4	64 ± 5.1	27.3 ± 1.2	2.3 ± 0.1	27.8 ± 2.2	20.7 ± 1.7	7.3 ± 0.5	34.9 ± 2.8	102 ± 9
Bromocriptine	20.7 ± 1.5	57.5 ± 3.5	78.2 ± 6.1	35.7 ± 2.5	6.9 ± 0.6	26.3 ± 1.5	19 ± 1.1	14.6 ± 1.2	45.1 ± 1*+	78 ± 6*+

* significantly different compared with control group ($p < 0.05$)
\+ significantly different compared with treated groups ($p < 0.05$)

Vvnu, Vvci and Vvce: Volume density of nuclei, cytoplasm and cell.
Vvmi and Vvly: Volume density of mitochondria and lysosome.
Sver, Svgo and Svmi: Surface density of endoplasmic reticulum, Golgi apparatus and mitochondria.
Nvgr and Dm: Numerical density and mean diameter of secretory granules.

Table 3. Electron microscopic morphometric analysis of densely granulated growth hormone cell adenoma treated for 4 h with TRH, SMMS 201-995 and bromocriptine *in vitro*.

	LEVEL I			LEVEL II		LEVEL III			AUTOMATIC IMAGE ANALYSIS	
	Vvnu	Vvci	Vvce	Vvmi	Vvly	Sver	Svgo	Svmi	Nvgr	Dm(nm)
Control	12.2 ± 1.1	28.5 ± 2.6	40.7 ± 3.4	28.2 ± 2.2	14.4 ± 1.1	31.1 ± 2.3	12.9 ± 0.6	19.1 ± 1.5	26.2 ± 1.3	303 ± 16
TRH	12.4 ± 1	36.3 ± 2.8	48.7 ± 4.5	53.7 ± 4*+	11.4 ± 1	43.3 ± 2.2*+	9.6 ± 0.7	25.1 ± 2.1	54.9 ± 3.7*	317 ± 20
SMS 201-995	18.4 ± 1.5	37.1 ± 3.5	55.5 ± 4.4	10.9 ± 0.9*	8.8 ± 0.6*	25.6 ± 1.4*	14.5 ± 1.3	14.2 ± 1.3	55.8 ± 2.4*	320 ± 18
Bromocriptine	18.7 ± 1.7	33.7 ± 2.5	51.7 ± 4.1	30.8 ± 2.3	22.4 ± 2.1	22.6 ± 2.1*	9.4 ± 0.9	12.5 ± 1.1*	55.5 ± 3.6*	325 ± 15

* significantly different compared with control group ($p < 0.05$)
\+ significantly different compared with treated groups ($p < 0.05$)

Vvnu, Vvci and Vvce: Volume density of nuclei, cytoplasm and cell.
Vvmi and Vvly: Volume density of mitochondria and lysosome.
Sver, Svgo and Svmi: Surface density of endoplasmic reticulum, Golgi apparatus and mitochondria.
Nvgr and Dm: Numerical density and mean diameter of secretory granules.

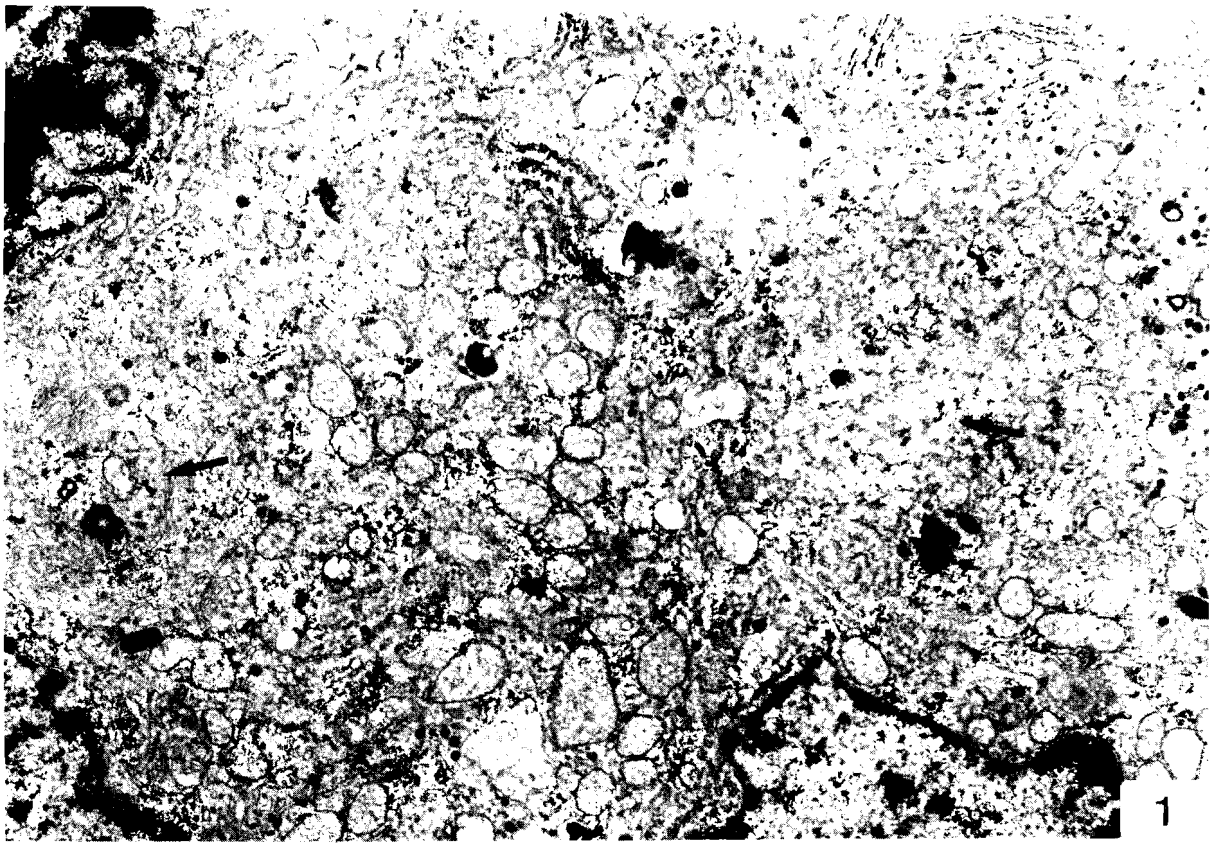


Fig. 1. Electron micrographs of sparsely granulated GH cell adenoma. Abundant and large mitochondria. Filamentous mass (arrows). Laminar RER. Peripherally arranged granules × 13,000

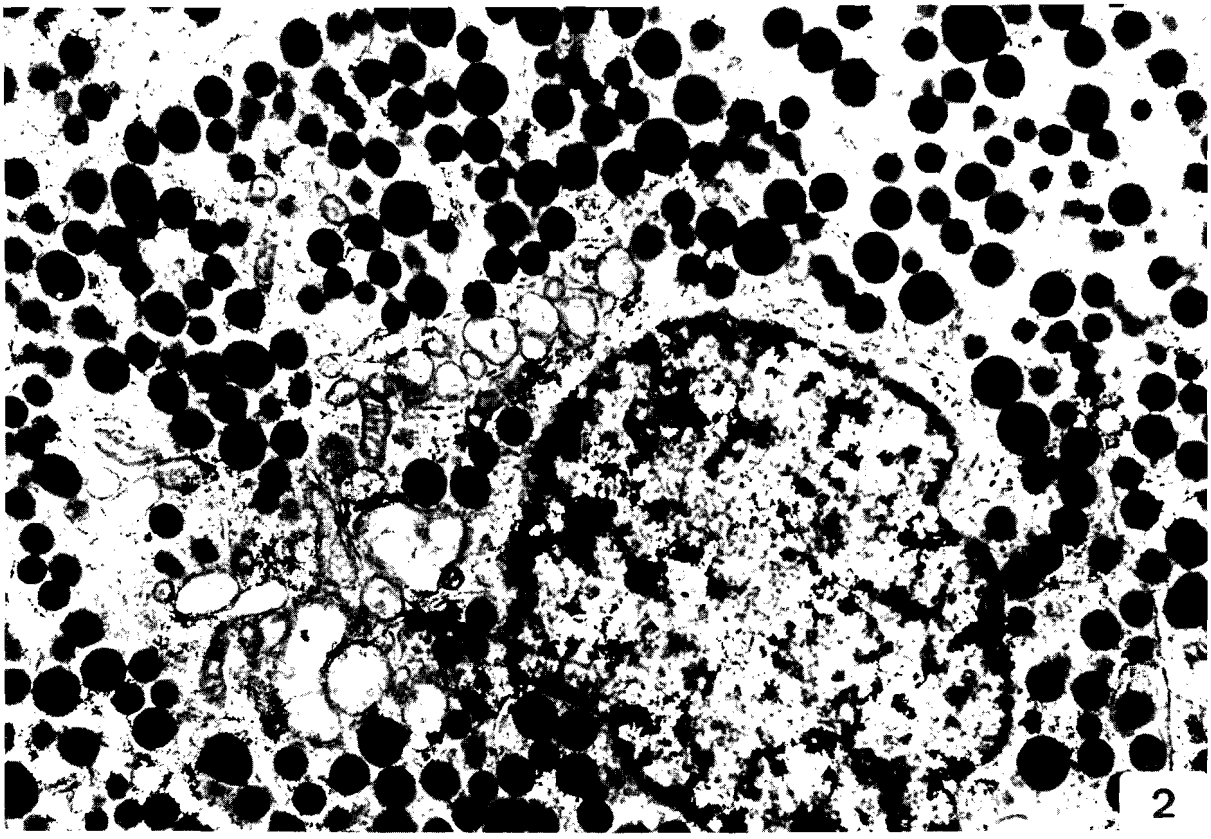
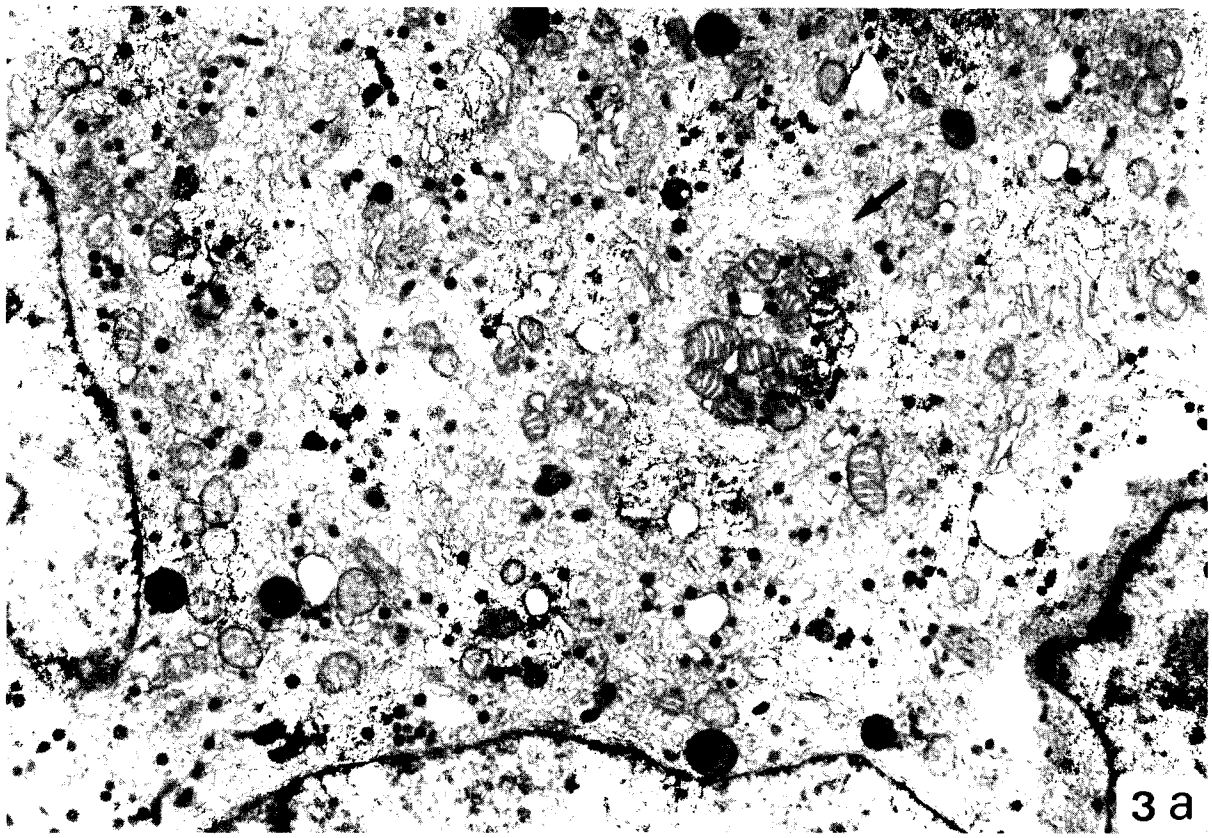
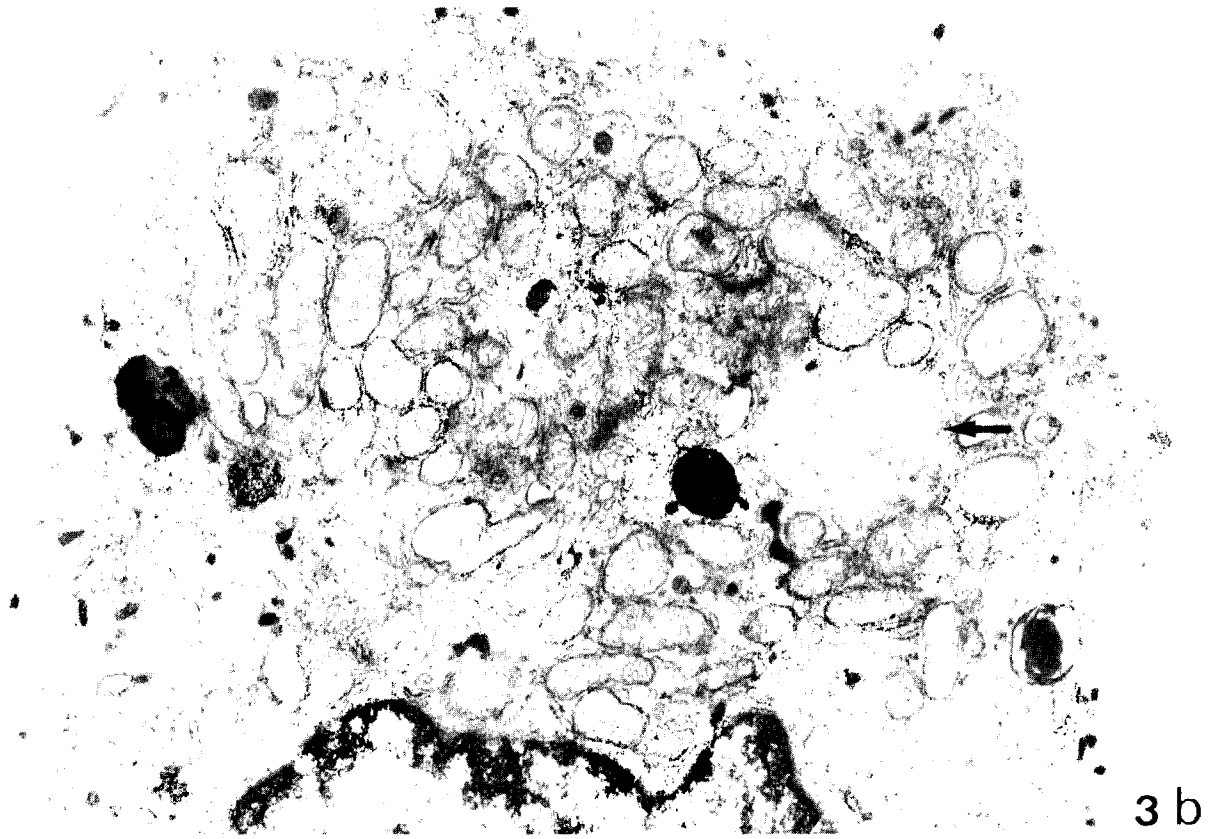
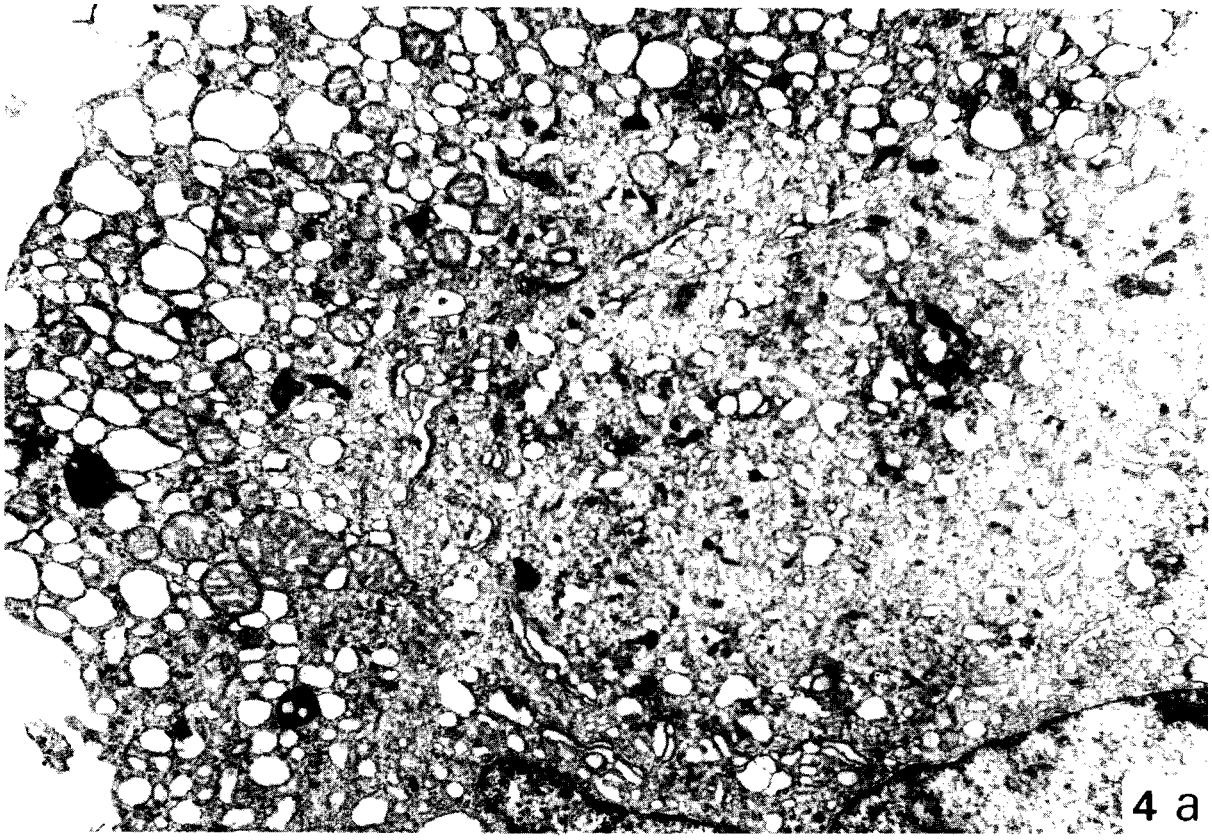


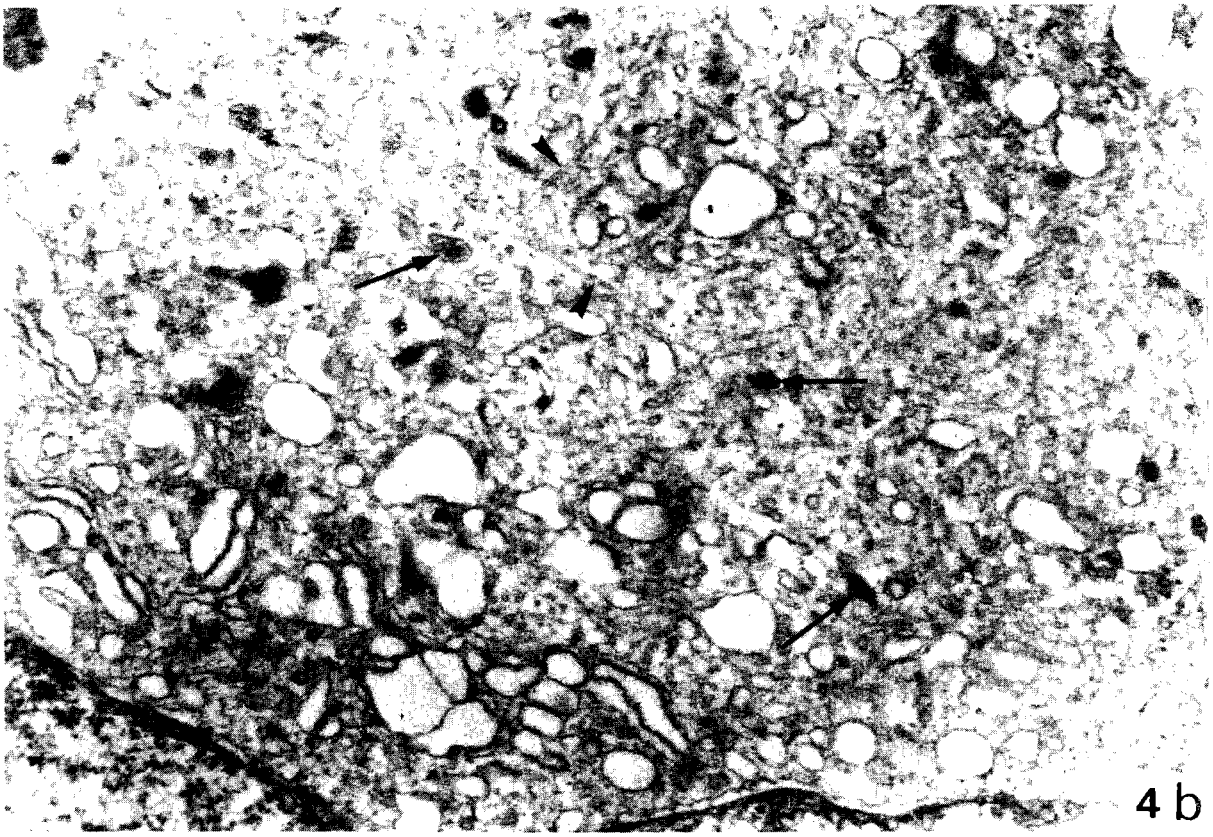
Fig. 2. Electron micrographs of densely granulated GH cell adenoma. Granules dispersed all over the cytoplasm. × 16,500



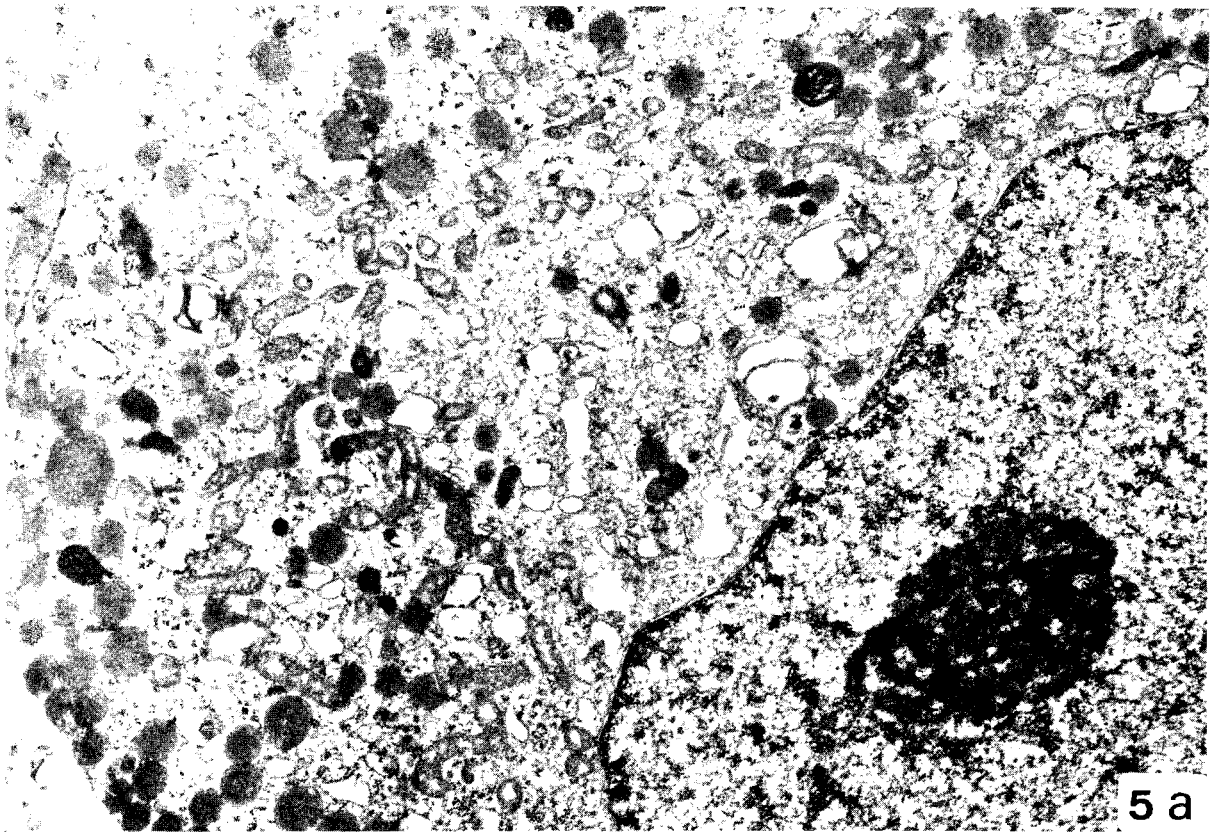


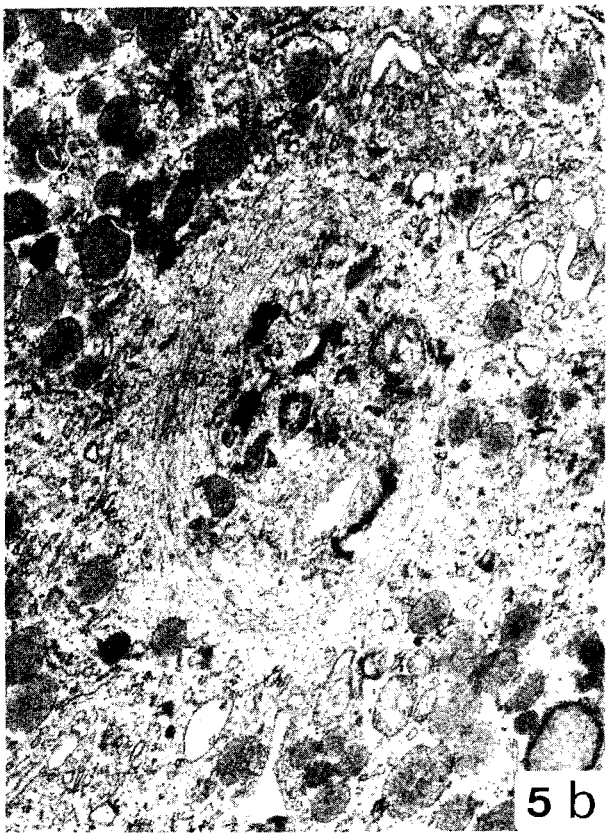
Figs. 3 a-b. 48 hour culture of sparsely granulated GH cell adenoma. **a)** Abundant mitochondria and manifest Golgi apparatus. Fibrous body with mitochondria and secretory granules inside (arrow) × 13,000. **b)** Fibrous body with abundant microfilaments. (arrow) × 23,650

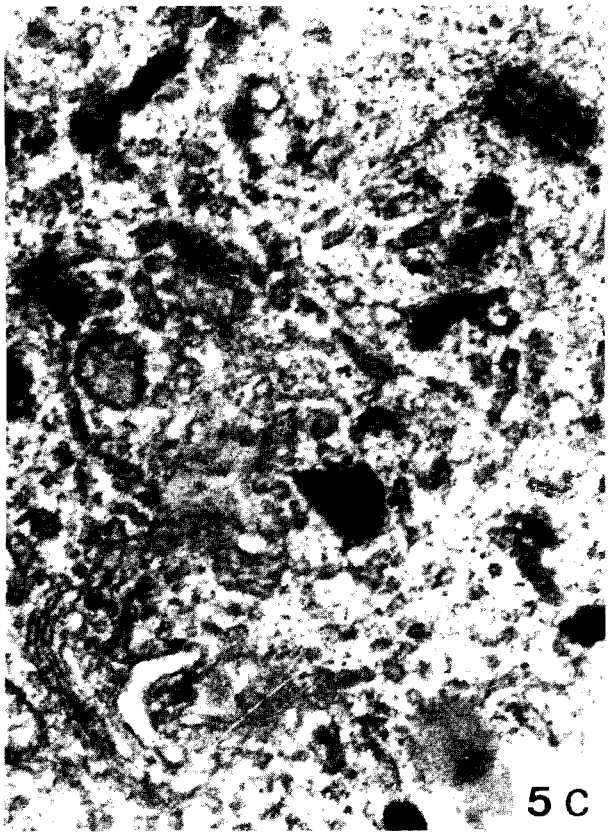




Figs 4a-b. Sparsely granulated GH cell adenoma treated for 4 hours with TRH. **a)** Wide Golgi area and dilatation of cisternae of RER × 14,000. **b)** Granules in formation (arrow) and microtubules (head arrow) × 36,000







Figs. 5a-c. Densely granulated GH cell adenoma, control culture. **a)** Abundant secretory granules and manifest Golgi apparatus × 20,000. **b)** Fibrous body × 28,000. **c)** Destructuring of Golgi apparatus and RER. Centriole × 50,000

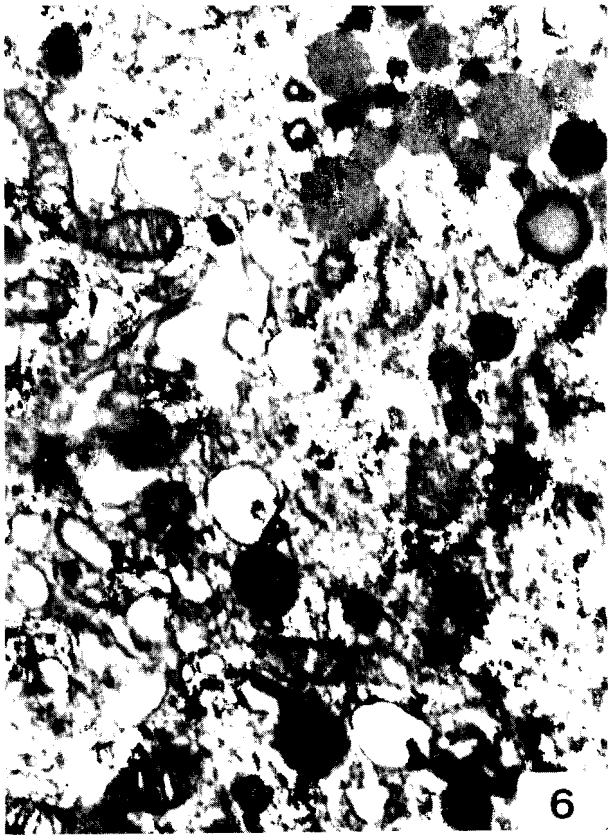


Fig. 6. Densely granulated GH cell adenoma treated for 4 hours with bromocriptine. Large and abundant granules with lysosomes in different formation stages. Images of crinophagy. × 30,000

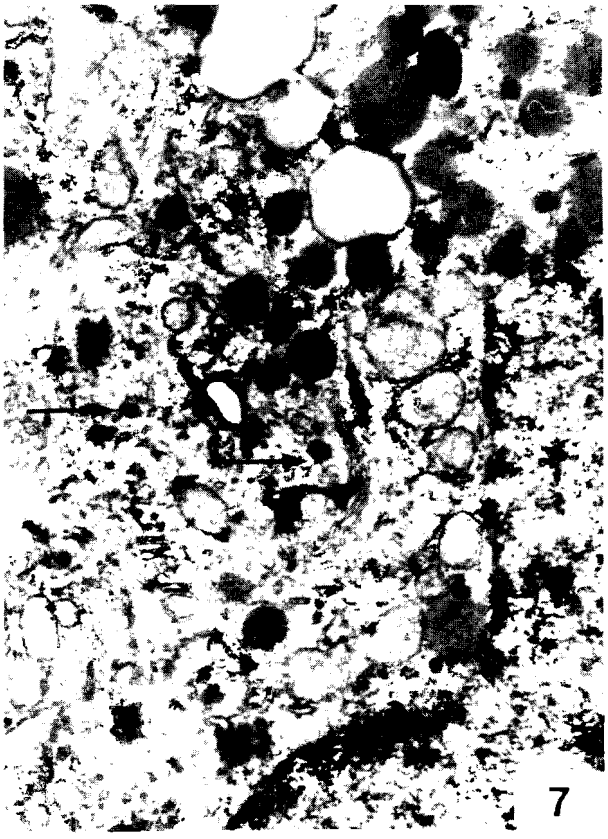


Fig. 7. Densely granulated GH cell adenoma treated with TRH for 4 hours. Granular packing (arrow). Crinophagy. × 25,000

formed by flattened cisternae and a Golgi apparatus was evident in nearly all cells. Secretory granules (Fig. 2) were very numerous and evenly scattered all over the cytoplasm with a diameter of 301 nm (Table 3). Considering its ultrastructural and immunocytochemistry characteristics we concluded it was a densely granulated GH cell adenoma.

2.2. In vitro

2.2.1 Sparsely granulated growth hormone cell adenoma

Control culture cells (Figs. 3a,b) presented similar characteristics to those of the specimen. Secretory granules with 107 nm mean diameter (Table 2) were scattered all over the cytoplasm and lysosomes were most easily noted (Fig. 3a). Several Golgi areas and a RER formed by small cisternae widely scattered all on the cytoplasm were frequently found. Mitochondria were very abundant and with inverted cristae (Fig. 3b). Fibrous bodies constituted only by microfilaments were observed (Fig. 3b) although they sometimes contained different cellular organelles (Fig. 3a). These structures were scarce in treated cultures.

Administration both of SMS 201-995 and of bromocriptine did not evidence overt ultrastructural changes with respect to controls.

Cells treated with TRH (Figs. 4a,b) presented a large Golgi apparatus (Fig. 4a) with granules in formation and microtubules (Fig. 4b). There was an evident dilation of RER cisternae (Fig. 4a).

2.2.2. Densely granulated growth hormone cell adenoma

In control cultures (Figs. 5a-c) cells had numerous secretory granules with 303 nm mean diameter (Table 3) and a conspicuous Golgi apparatus (Fig. 5a). Fibrous bodies consisting of numerous microfilaments that had entrapped secretory granules, mitochondria and centrioles appeared in the Golgi area of some cells (Fig. 5b). In some cytoplasmic areas (Fig. 5c) where one or several centrioles usually appear, different organelles were gathered together, mainly Golgi sacules and RER cisternae. As we moved towards the center of these areas, that resembled fibrous bodies, we observed destructuring of these organelles.

Treatment with SMS 201-995 and bromocriptine (Fig. 6) showed the existence of abundant secretory granules

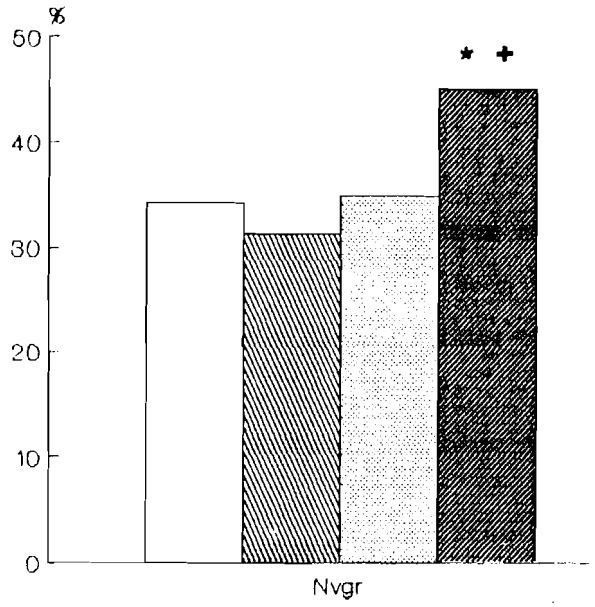


FIGURA 8a

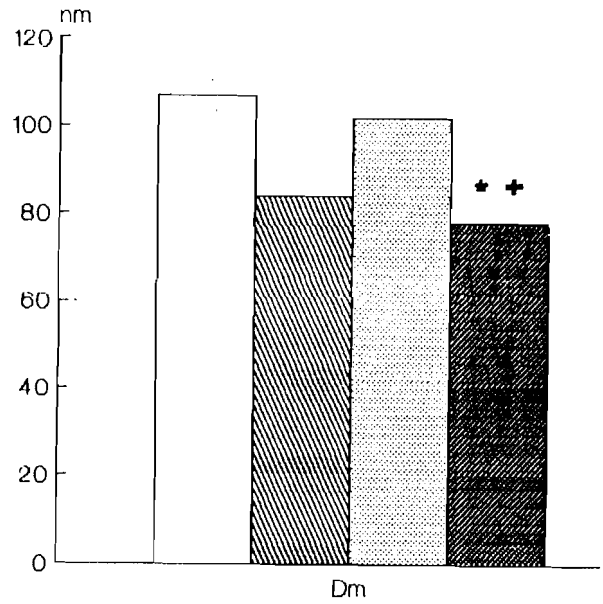
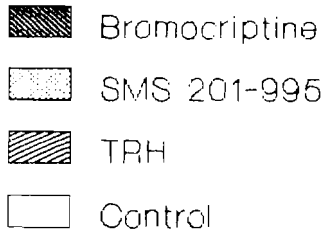


FIGURA 8b



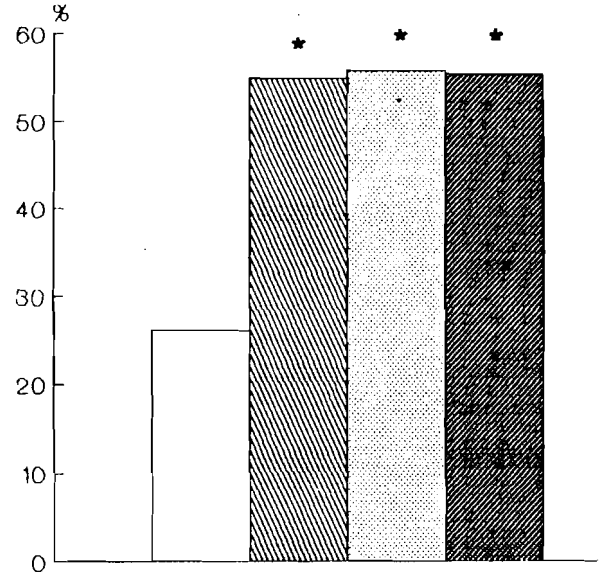


FIGURA 8c

Figs. 8a-e. a) Numerical density of secretory granules (Nvgr) from sparsely granulated GH cell adenoma. **b)** Mean diameter of secretory granules (Dm) from sparsely granulated GH cell adenoma. **c)** Numerical density of secretory granules (Nvgr) from densely granulated GH cell adenoma. **d)** Volume densities of mitochondria (Vvmi) and lysosomes (Vvly) from densely granulated GH cell adenoma. **e).** Surface densities of endoplasmic reticulum (Sver) and mitochondria (Svmi) from densely granulated GH cell adenoma.
* significantly different compared with control group ($p < 0.05$).
+ significantly different compard with treated groups ($p < 0.05$).

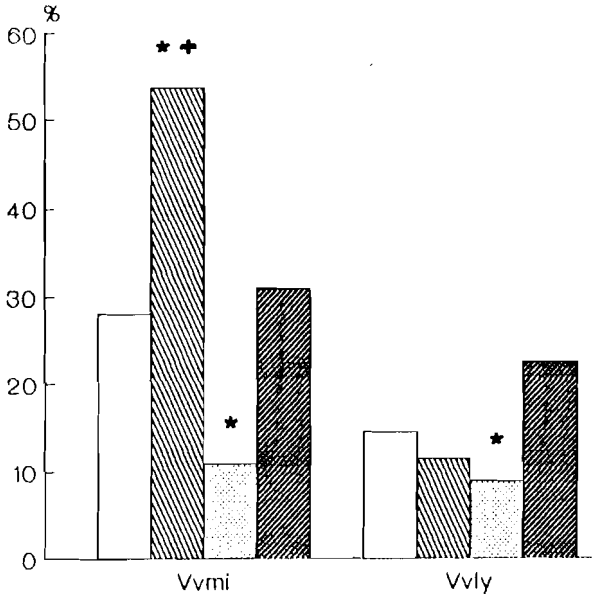


FIGURA 8d

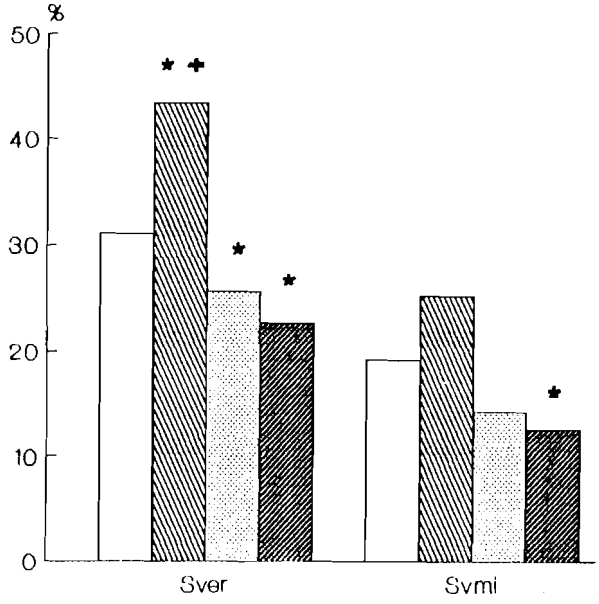


FIGURA 8e

and lysosomial formations at different levels of evolution. Crinophagy was frequently present.

TRH presented no great ultrastructural differences with respect to case 1. Granular packing was conspicuous; lysosomes at different levels of evolution and crinophagy appeared in the Golgi apparatus (Fig. 7).

3. Morphometry

Morphometric data are summarized in tables 2 and 3, which show the mean values (±) and standard error (SE).

In the sparsely granulated GH cell adenoma (Table 2), bromocriptine increased numerical density of secretory granules (Nvgr) (Fig. 8a) and decreased the mean diameter of the secretory granules (Dm) (Fig. 8b) with respect to the other cultures. SMS 201-995 caused not significant variations. TRH increased surface density of RER (Sver) with respect to the other cultures.

In the densely granulated GH cell adenoma (Table 3), SMS 201-995 and bromocriptine increased the Nvgr with respect to control (Fig. 8c). SMS 201-995 decreased mitochondrial volume density (Vvmi) and lysosomial volume density (Vvly) (Fig. 8d) and Sver (Fig. 8e) with respect to control. Bromocriptine decreased Sver and Svmi (Fig. 8e) with respect to control.

TRH increased the Nvgr (Fig. 8c) with respect to control. It also increased Vvmi (Fig. 8d) and Sver (Fig. 8e) with respect to the other cultures.

Discussion

In the present study we investigated by immunocytochemistry, electron microscopy and morphometry, the acute effects (4 hours) induced by TRH, SMS 201-995 and bromocriptine in two GH-producing pituitary adenomas *in vitro*.

Considering their immunocytochemical and ultrastructural characteristics, these adenomas are from densely and sparsely granulated cells (Oliver et al., 1975; Kovacs and Horvath, 1986).

The most significant data of our study can be summarized as follows: 1) The most evident changes, shown by morphometry, refer to the secretory granules, mitochondria and RER. 2) In both adenomas, TRH increased the Sver with respect to the other cultures. 3) SMS 201-995 and bromocriptine had different effects according to GH adenoma cell type. 4) Presence *in vitro* of fibrous bodies in cells of both types of adenomas was constant.

At 48 hours, cells kept in culture presented morphological and immunocytochemical characteristics similar to those of the tumor specimen. Longer culture periods determine cellular changes that mainly affect the secretory granules, RER and lysosomes (Loras et al., 1985). We consequently studied the acute effect of TRH, SMS 201-995 and bromocriptine at 48 hours and not following longer periods that produce cellular changes inherent in the evolution of the culture.

TRH *in vivo* produces a paradoxical effect of GH secretion in acromegalic adenomas (Marcovitz et al., 1982). This effect does not occur in the densely granulated GH cell adenoma, where serum levels decreased instead (58.8 ng/ml at 40.9 ng/ml). In this adenoma, TRH caused an increase of Sver, Vvmi and Nvgr *in vitro*, proving the presence of hormonal synthesis.

In the sparsely granulated GH cell adenoma, TRH produces only morphological changes of Sver, suggesting a different response of these two adenomas to TRH *in vitro*.

In vivo administration of SMS 201-995 (Plewe et al., 1984; Lamberts et al., 1985a; George et al., 1987) and bromocriptine (Peillon et al., 1979; Spada et al., 1982; Opizzi et al., 1984; Lamberts et al., 1985b) decreases GH serum levels. In the densely granulated GH cell adenoma, GH serum levels decreased more with 100 µg SMS 201-995/subcutaneous (31.6 ng/ml at 8.1 ng/ml) than with 5 mg bromocriptine/oral (84 ng/ml at 24 ng/ml). These values were not measured in the sparsely granulated GH cell adenoma. *In vitro*, SMS 201-995 and bromocriptine increased the Nvgr in the densely granulated GH cell adenoma, similar alterations being described by Peillon et al. (1979). Moreover, SMS 201-995 decreased Vvmi, Vvly and Sver and thus clearly inhibits the hormonal synthesis.

In the sparsely granulated GH cell adenoma, SMS 201-995 did not produce significant changes, whereas bromocriptine increased the Nvgr and decreased Dm. In this case, lack of response to SMS 201-995 *in vitro* could be due to the presence of GH-producing adenomas with a low density of receptor for somatostatin (Reubi et al., 1987), probably owing to desensitization due to high levels of GH serum (Moyse et al., 1985).

In both adenoma cells, changes produced by SMS 201-995 and by bromocriptine affect mainly the morphometric parameters related to secretory granules, which is in agreement with the findings of Peillon et al. (1976) with somatostatin *in vitro* and George et al. (1987) with SMS 201-995 *in vivo*.

Presence of intracellular fibrous bodies is associated with GH-producing adenomas and they do not appear in other pituitary adenomas (Roy, 1978). In the sparsely granulated GH cell adenomas, their presencce is constant, appearing only in 20% of the densely granulated GH cell adenomas (Horvath and Kovacs, 1976). In our study we noted presence of fibrous bodies in both adenomas *in vitro*, while *in vivo* they only appeared in the densely granulated GH cell adenoma. On the contrary, we observed some intracellular formations that we interpreted as fibrous bodies in different stages of evolution.

In summary, cellular changes produced *in vitro* by TRH, SMS 201-995 and bromocriptine in short periods of time (4 hours) in GH-producing pituitary adenomas suggest different responses according to adenoma cell types. In densely granulated GH cell adenoma, the morphological changes remind one of those wich appear with TRH, SMS 201-995 and bromocriptine in normal human somatotroph cells (Marcovitz et al., 1982).

Nevertheless, morphological changes are only evident by morphometric study as ultrastructure alone is not able to present the necessary data to prove these changes.

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